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Regulation of Angiotensin II-stimulated Ca^{2+} Oscillations by Ca^{2+} Influx Mechanisms in Adrenal Glomerulosa Cells*

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In adrenal glomerulosa cells, angiotensin II (Ang II) evokes repetitive $[Ca^{2+}]_i$ transients and increases Ca^{2+} influx through voltage-sensitive calcium channels (VSCCs) as well as the capacitative Ca^{2+} entry pathway. This study analyzed the relationships between these Ca^{2+} influx pathways and intracellular Ca^{2+} signaling in bovine glomerulosa cells, in which Ca^{2+} oscillation frequency was regulated by Ang II concentration over the range of 50–300 pM. In the absence of external Ca^{2+}, such oscillations were maintained for prolonged periods of time, but their frequency was significantly reduced (0.23 min^{-1} *versus* 0.38 min^{-1}). Restoration of $[Ca^{2+}]_o$ to 0.6 mM increased the frequency of Ca^{2+} oscillations in cells that showed narrow spikes of constant amplitude and caused a plateau response in cells with broad spikes of rapidly decreasing amplitude. In the presence of Ca^{2+}, nifedipine reduced the frequency of the oscillatory Ca^{2+} response to 100 pM Ang II by 49%, and BAY K 8644 increased oscillation frequency by 86%, or caused plateau-type responses typical of higher Ang II concentrations. In contrast to their prominent actions on Ca^{2+} spiking frequency, dihydropyridines caused only minor changes in Ang II (100 pM)-induced inositol phosphate production. Dihydropyridines also had minimal effects on the nonoscillatory Ca^{2+} signals evoked by high Ang II concentrations (10 nM). These findings indicate that Ca^{2+} influx through VSCCs modulates the frequency of Ca^{2+} oscillations induced by low agonist concentrations by a mechanism that does not involve major changes in inositol trisphosphate formation. However, VSCCs make relatively little contribution to the nonoscillatory Ca^{2+} signals generated by high agonist concentrations, when Ca^{2+} influx occurs predominantly through the capacitative Ca^{2+} entry pathway.

Repetitive Ca^{2+} transients occur in a variety of cell types following stimulation with low concentrations of Ca^{2+}-mobilizing agonists. This type of Ca^{2+} signaling consists of a series of Ca^{2+} spikes that occur at intervals of 0.5–5 min, depending on the agonist dose and the cell type. Such oscillatory signals, which often persist for 1 h or more, have been observed in cell types ranging from electrically nonexcitable cells such as hepatocytes through partially excitable endocrine and neuroendocrine cells (*e.g.* pancreatic β cells, pituitary cells, and adrenal glomerulosa cells) to classical excitable cells (muscle cells, neurons) (1). In many cases the spiking frequency, rather than the amplitude of the oscillations, is regulated by the agonist concentration, providing the basis of a frequency-encoded Ca^{2+} signaling mechanism. Although Ca^{2+} release from internal stores is a major source of the cytoplasmic Ca^{2+} transients, $[Ca^{2+}]_i$ levels are also influenced by Ca^{2+} entry mechanisms operating through plasma membrane Ca^{2+} channels. These include a ubiquitous form of Ca^{2+} influx in which the filling state of the Ca^{2+} stores regulates Ca^{2+} influx ("capacitative Ca^{2+} entry"; see Ref. 2) as well as various types of voltage-sensitive calcium channels (VSCCs)[1] that determine Ca^{2+} influx as a function of the membrane potential in excitable cells.

Recent models to explain the generation of Ca^{2+} oscillations have focused on the dual regulation of the $InsP_3$ receptor channel by its ambient cytosolic Ca^{2+} concentrations, with positive and negative feedback by low and high $[Ca^{2+}]_i$, respectively. This mechanism has been proposed to initiate and terminate short bursts of Ca^{2+} release even at constant $InsP_3$ levels (3–5). The restoration of basal $[Ca^{2+}]_i$ between spikes depends on diverse Ca^{2+} homeostatic mechanisms, including Ca^{2+} pumps located in the endoplasmic reticulum (ER) and plasma membrane (6, 7). These processes, and possibly uptake by mitochondria (7–10), reestablish the resting $[Ca^{2+}]_i$ levels and also refill the Ca^{2+} pools so that a new cycle can be initiated. Due to the high Ca^{2+} sensitivity of the $InsP_3$ receptor channel, disturbances in the Ca^{2+} homeostatic mechanism(s) of the cell frequently affect the rate and character of Ca^{2+} oscillations. For example, the Ca^{2+} wave frequency in *Xenopus* oocytes can be accelerated by enhancement of Ca^{2+} influx (11) as well as by overexpression of a SERCA type Ca^{2+} pump to promote the rapid resetting of basal $[Ca^{2+}]_i$ levels (12). These findings indicate the involvement of both Ca^{2+} entry and reuptake mechanisms in the regulation of periodic calcium signaling in these large cells. Recent microfluorimetric studies revealed that small microdomains, or so-called hot spots, with little impact on the average $[Ca^{2+}]_i$ of the cell may have a crucial role in the regulation of intracellular Ca^{2+} signaling events (9, 13). This implies that if the Ca^{2+} stores with their $InsP_3$ receptors and the plasma membrane Ca^{2+} entry channels are close to one another (14), even small amounts of Ca^{2+} entering through these channels could be an important factor in the regulation of Ca^{2+} signals by the local effects of Ca^{2+} influx.

In the adrenal glomerulosa, the presence of both the capacitative Ca^{2+} entry pathway (15, 16) and various types of VSCC (17–21) have been reported. Both of these Ca^{2+} influx mechanisms are active during Ang II stimulation of bovine glomerulosa cells (15, 22) providing two complementary means to regulate intracellular Ca^{2+} signaling through Ca^{2+} entry. Thus,

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[1] The abbreviations used are: VSCC, voltage-sensitive calcium channels; $InsP_3$, inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; Ang II, angiotensin II; DHP, dihydropyridine(s); KRBS, Krebs-Ringer buffer solution.

dihydropyridines have been shown to exert minor but consistent effects on the $[Ca^{2+}]_i$ plateau following stimulation with high doses of Ang II in suspensions of adrenal zona glomerulosa cells (22–24). Such results have suggested that voltage-dependent Ca^{2+} channels make relatively little contribution to the Ca^{2+} signals generated by Ang II in adrenal cells, which contrasts with the sensitivity of the secretory response of these cells to dihydropyridines, especially at low Ang II concentrations (16). This apparent controversy could be resolved, if the role of VSCC activation is not to increase the overall cytosolic Ca^{2+} levels but rather to modulate Ca^{2+} oscillations that occur primarily at lower, more physiological doses (25, 26). To explore the relationship between Ca^{2+} entry through VSCC and Ca^{2+} oscillations, we examined the relative contributions of the two Ca^{2+} entry pathways (VSCC and capacitative Ca^{2+} influx) to the regulation of periodic Ca^{2+} release from internal Ca^{2+} stores in Ang II-stimulated bovine adrenal glomerulosa cells.

EXPERIMENTAL PROCEDURES

Materials—Fura-2/AM, Indo-2/AM, and pluronic acid were purchased from Molecular Probes, Inc. (Eugene, OR). Angiotensin II was purchased from Peninsula Laboratories, Inc. (San Carlos, CA). BAY K 8644, nifedipine, and thapsigargin were obtained from Calbiochem. All other chemicals were from Sigma. Cell culture media were prepared by the NIH Media Unit (Bethesda, MD) or were supplied by Biofluids (Rockville, MD).

Cell Culture—Bovine adrenal zona glomerulosa cells were prepared and cultured as described previously (27). Cells were plated on poly-L-lysine-coated circular coverslips placed in the individual wells of 6-well culture dishes (Corning, NY) at a density of 10^5 cells/ml (3 ml of cell suspension/well). Cells were cultured for 2–5 days in Dulbecco's modified Eagle's medium supplemented with donor horse serum (10%) and fetal bovine serum (2%) before Ca^{2+} measurements.

Cytosolic $[Ca^{2+}]$ Measurements—Cells attached to coverslips were loaded with 0.5–5 μM fura-2/AM or indo-1/AM for 2–5 h at room temperature in a modified Krebs-Ringer buffer solution (KRBS) containing 118 mM NaCl, 2.42 mM KCl, 1.8 mM Ca^{2+}, 1.18 mM KH_2PO_4, 0.8 mM $MgSO_4$, 20 mM Na-Hepes (pH 7.4), 5 mM $NaHCO_3$, 10 mM glucose, and 1 mg/ml bovine serum albumin, supplemented with 0.25% pluronic acid. For $[Ca^{2+}]_i$ measurements, the loading buffer was replaced by fresh KRBS or Ca^{2+}-free KRBS. The coverslips were then placed in a 35-mm diameter Teflon culture dish (Medical Systems Corporation, Greenvale, NY), and the $[Ca^{2+}]_i$ was determined by microfluorometry using either a single excitation, two emission wavelength procedure with indo-1 (28), or a dual excitation single emission wavelength protocol with fura-2 (29). All stimulations and pharmacological treatments of the cells were performed at room temperature by the addition of the substance diluted in 1 ml of buffer.

Inositol Phosphate Measurements—Inositol phosphates were measured in glomerulosa cells, prelabeled with *myo*-[^{3}H]inositol (20 μCi/ml) for 24 h, after extraction and separation by high pressure liquid chromatography as described previously (24). Because of the small inositol phosphate response of the cells to 100 pM Ang II, cells were preincubated with LiCl (10 mM) for 30 min and were stimulated with Ang II for 30 min to capture the metabolites of inositol 1,4,5-trisphosphate. Experiments were also performed without lithium, which showed similar results but with smaller changes.

Statistical Analysis—Values are given as mean ± S.E. Analyses of statistical significance were performed using paired or unpaired *t* tests. For multiple comparisons we used analysis of variance combined with the Duncan test.

RESULTS

Properties of Single Cell $[Ca^{2+}]_i$ Responses of Glomerulosa Cells—In bovine adrenal zona glomerulosa cells, stimulation with 100 pM Ang II induced repetitive $[Ca^{2+}]_i$ transients with an average frequency of 0.38 ± 0.03 min^{-1} (n = 40 cells, Fig. 1*A*). The cytosolic Ca^{2+} concentration returned to basal levels between individual spikes, which were usually maintained for >1 h with only a slight decrease in amplitude. A 3-fold increase in Ang II concentration caused a significant increase in oscillatory frequency from 0.38 ± 0.05 to 0.63 ± 0.09 min^{-1} ($p < 0.005$, n = 5 cells, Fig. 1*A*) with no major change in spike amplitude. The subsequent addition of 1 nM Ang II elicited a large peak followed by a sustained plateau (Fig. 1*B*). Direct application of higher concentrations of Ang II (10 nM) usually induced similar biphasic Ca^{2+} responses with an initial spike followed by a plateau (n = 12, Fig. 1*C*), but in a subset of cells (35%) it elicited rapid Ca^{2+} oscillations. This oscillatory signal was preceded by an initial rise in $[Ca^{2+}]_i$ with rapid superimposed oscillations of irregular amplitude and an average frequency of 2.07 ± 0.40 min^{-1} (n = 4, Fig. 1*D*). These high frequency spikes often merged to resemble the early phase of a sustained plateau response (n = 3, Fig. 1*E*). After a few such peaks the signal switched to regular base-line spiking with a frequency of 1.36 ± 0.25 min^{-1} (n = 7, Fig. 1, *D* and *E*). The responsiveness of individual cells showed some variation, and plateau elevations in $[Ca^{2+}]_i$ were occasionally observed even with 100 pM Ang II. Regular oscillations of very low frequency could be elicited with Ang II concentrations as low as 25 pM, which is within the physiological range of the octapeptide present in the circulation (data not shown).

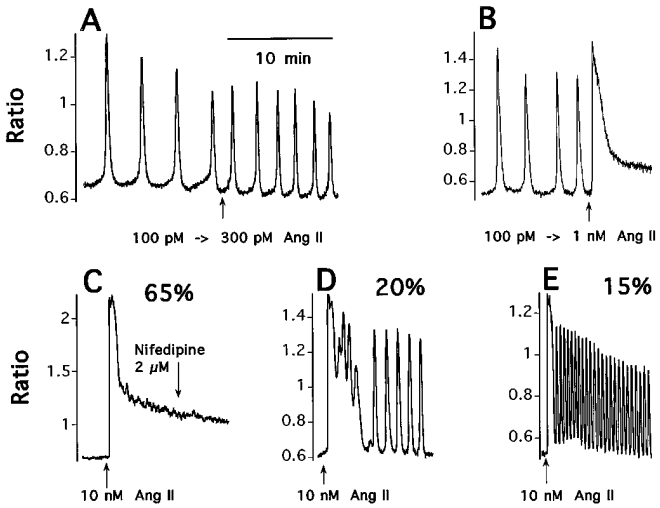

FIG. 1. **Concentration dependence of the $[Ca^{2+}]_i$ response to stimulation with Ang II in single bovine adrenal zona glomerulosa cells.** *A*, Ang II (100 pM) induces repetitive Ca^{2+} transients in the adrenal glomerulosa cell. A 3-fold increase in the agonist concentration causes an increase in the frequency of the Ca^{2+} oscillation. *B*, when the concentration of Ang II is raised to 1 nM, the cell responds with a large transient $[Ca^{2+}]_i$ increase followed by a sustained plateau. *C*, a typical "biphasic" response consisting of a sharp initial rise in $[Ca^{2+}]_i$ followed by a plateau elevation usually occurs after exposure to 10 nM Ang II (65% of all cells tested). Such a plateau phase is not affected by nifedipine. *D* and *E*, in about one-third of the cells (35%), Ca^{2+} oscillations were observed following stimulation with 10 nM Ang II as well. This oscillatory signal is typically of high frequency and is preceded by rapid irregular (*D*) or merged (*E*) calcium spikes.

Role of Ca^{2+} Influx in the Modulation of Ca^{2+} Oscillations Induced by Low Agonist Concentrations—To study the role of extracellular Ca^{2+} in the regulation of Ca^{2+} oscillations, glomerulosa cells were stimulated with 100 pM Ang II in Ca^{2+}-free medium (no added Ca^{2+}). Under these conditions, sustained Ca^{2+} oscillations were again evident for >1 h in most of the cells. The frequency of these sustained oscillations (but not their amplitude) was significantly lower than that observed in the presence of extracellular Ca^{2+} (0.23 ± 0.01 min^{-1}, n = 45, $p < 0.001$).

The addition of 0.6 mM Ca^{2+} to such Ang II-stimulated cells immediately increased the oscillatory frequency from 0.27 ± 0.02 to 0.52 ± 0.06 min^{-1} (n = 16 cells, $p < 0.001$, Fig. 2*A*). Subsequent increases of $[Ca^{2+}]_o$ to 1.2 mM or higher did not cause any further increase in frequency (data not shown). In a subset of cells, addition of extracellular Ca^{2+} caused an immediate spike followed by a plateau Ca^{2+} elevation (n = 16, Fig.

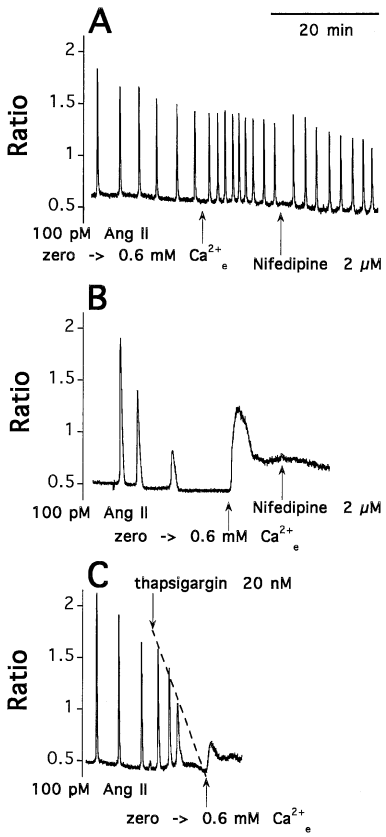

FIG. 2. **Role of Ca^{2+} entry in the generation of repetitive Ca^{2+} transients evoked by 100 pM Ang II.** *A* and *B* show typical examples of cells that exhibit oscillatory Ca^{2+} signaling after stimulation with 100 pM Ang II in the absence of extracellular Ca^{2+}. The addition of 0.6 mM $[Ca^{2+}]_o$ (while keeping the Ang II concentration constant) leads to an increase in frequency in cells that display narrow Ca^{2+} transients of stable amplitude ("synchronized" type (*A*)). In some cells that show wide spikes with rapidly decreasing amplitudes, Ca^{2+} addition leads to a plateau type of response ("non-synchronized" (*B*)), similar to the effects of increasing agonist doses (see Fig. 1*B*). The addition of nifedipine partially reverses the frequency increase in *A* (see also Table II). However, once a plateau is reached nifedipine is unable to restore base-line spiking (*B*), suggesting the dominance of the capacitative non-DHP-sensitive component in this type of response. A low concentration of thapsigargin (20 nM) causes a transient increase in frequency, with loss of spike amplitude and widening of the Ca^{2+} spikes before the oscillations cease. The addition of extracellular Ca^{2+} in such thapsigargin-treated cells always leads to a relatively low biphasic rise in $[Ca^{2+}]_i$ (*C*).

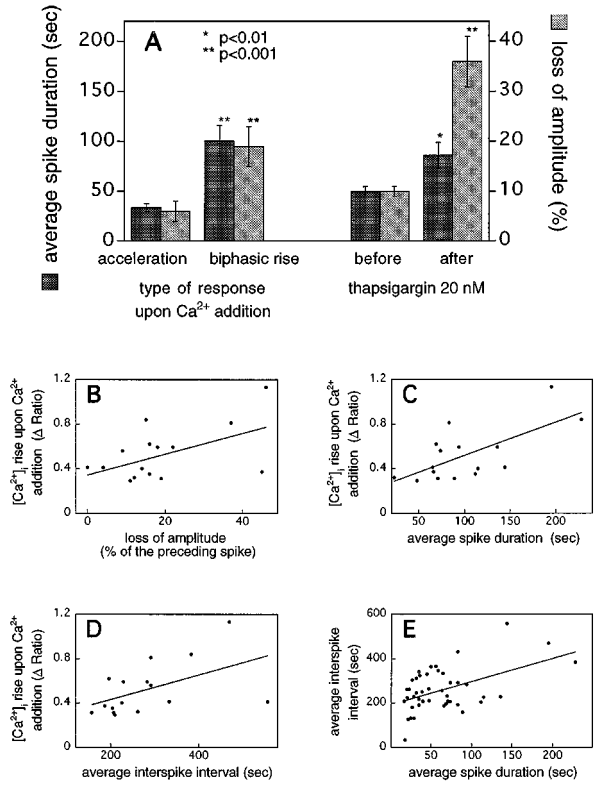

FIG. 3. **Correlation between Ca^{2+} oscillatory parameters in Ca^{2+}-free medium and the type of Ca^{2+} response to the addition of extracellular Ca^{2+}.** *A*, adrenal glomerulosa cells that were stimulated with 100 pM Ang II in Ca^{2+}-free medium responded to the addition of 0.6 mM Ca^{2+} either with a frequency increase or with a biphasic Ca^{2+} response. Cells that responded with increased frequency had significantly shorter spike duration and little loss of spike amplitude before the Ca^{2+} addition, while the biphasic Ca^{2+} response was characteristic of cells that had longer spike duration and rapid loss of amplitude in Ca^{2+}-free medium. Thapsigargin (20 nM) was able to increase spike duration and caused pronounced loss of spike amplitude, thereby mimicking the pattern of the cells showing biphasic response. *Panels B–D*, correlations between oscillatory parameters observed in Ca^{2+}-free medium and the $[Ca^{2+}]_i$ rise detected after Ca^{2+} addition in cells showing biphasic $[Ca^{2+}]_i$ responses after restoring external Ca^{2+} ($p < 0.05$ (*B*); $p < 0.01$ (*C*); $p < 0.05$ (*D*)). *E*, correlation between spike duration and oscillatory frequency in cells stimulated with 100 pM Ang II in Ca^{2+}-free medium ($n = 45$, $p < 0.01$).

2*B*). Interestingly, such cells showed a pronounced decrease in the amplitude of their Ca^{2+} transients during stimulation in Ca^{2+}-free medium (Fig. 2*B*). The average decrease in spike amplitude (19 ± 3% per spike) was significantly higher in this group of cells than in cells that continued to oscillate after the addition of Ca^{2+} (6 ± 2%, $n = 16$, $p < 0.001$, Fig. 3*A*). The loss of spiking amplitude observed in Ca^{2+}-free medium was positively correlated with the magnitude of the $[Ca^{2+}]_i$ rise after the addition of Ca^{2+} (Fig. 3*B*, linear fit, $p < 0.05$). The amplitude of the biphasic $[Ca^{2+}]_i$ increase upon addition of Ca^{2+} also correlated with the average width of the Ca^{2+} spikes observed in Ca^{2+}-free medium (Fig. 3*C*, linear fit, $p < 0.01$), as well as with the interspike interval length (Fig. 3*D*, linear fit, $p < 0.05$). The average spike duration was usually significantly higher (101 ± 13 s, $n = 16$) in cells that responded with a biphasic Ca^{2+} increase when the $[Ca^{2+}]_o$ was increased fom 0 to 0.6 mM than in those that continued to oscillate after the readdition of Ca^{2+} (34 ± 4 s, $n = 16$, $p < 0.001$, Fig. 3*A*). There was a significant correlation between the average spike duration and the average interspike interval, based on the data from all cells stimulated with 100 pM Ang II in Ca^{2+}-free medium (Fig. 3*E*, linear fit, $p < 0.01$). That is, the shorter the Ca^{2+} spike duration, the more rapid were the oscillations.

Effect of Thapsigargin on Ang II-induced Ca^{2+} Oscillations— The above correlation between the average duration of $[Ca^{2+}]_i$ spikes and the oscillatory frequency was relevant to recent observations in *Xenopus laevis* oocytes, in which overexpression of a SERCA Ca-ATPase caused an increase in the frequency of $InsP_3$-induced $[Ca^{2+}]_i$ waves and a decrease in the average wave duration (12). The effect of changes in SERCA Ca^{2+}-ATPase activity on Ca^{2+} oscillations in glomerulosa cells during stimulation with 100 pM Ang II in Ca^{2+}-free medium was evaluated by treatment with low concentrations of the microsomal Ca^{2+}-ATPase inhibitor, thapsigargin. The impairment of Ca^{2+} sequestering ability caused by the addition of 20 nM thapsigargin to cells exhibiting narrow Ca^{2+} spikes caused an increase in spike duration and a more rapid loss of amplitude (Fig. 2*C*). This effect resembled the oscillatory pattern of cells that responded with biphasic Ca^{2+} increases after the addition of Ca^{2+} (Fig. 3*A* and Table I). Such thapsigargin-treated cells always displayed a biphasic rise in $[Ca^{2+}]_i$ upon addition of 0.6 mM $[Ca^{2+}]_o$ and never continued to oscillate ($n = 13$, Fig. 2*C*). However, it should be noted that the mean amplitude of the rise in $[Ca^{2+}]_i$ in response to extracellular Ca^{2+} was

TABLE I

Ca^{2+} oscillations elicited by low (100 pM) and high (10 nM) concentrations of Ang II in Ca^{2+}-free medium

Single glomerulosa cells were stimulated in Ca^{2+}-free medium with the indicated concentrations of Ang II. Once an oscillatory $[Ca^{2+}]_i$ signal had been established, 0.6 mM $[Ca^{2+}]_o$ was added to the medium. At both Ang II concentrations, two types of responses were observed upon addition of extracellular Ca^{2+} ions. Cells stimulated with 100 pM Ang II displayed either an acceleration in their oscillatory frequency or a biphasic $[Ca^{2+}]_i$ increase. Treatment of cells that belonged to the former group with thapsigargin (20 nM) caused a change to an oscillatory pattern that was characteristic of the latter group and also made these cells to respond with biphasic $[Ca^{2+}]_i$ increase after Ca^{2+} addition. Ca^{2+} oscillations driven by 10 nM Ang II either were independent of extracellular Ca^{2+} or showed a biphasic rise in $[Ca^{2+}]_i$. The latter group was similar in its parameters to the cells that responded with a biphasic Ca^{2+} increase upon addition of Ca^{2+} after stimulation with 100 pM Ang II. The former group, however, resembled in its spike parameters the one that was stimulated by 100 pM Ang II and was accelerated by external Ca^{2+}]. (*, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.001$ compared with the amplitude observed in the absence of thapsigargin).

	100 pM Ang II				10 nM Ang II	
	Acceleration of spiking frequency ($n = 16$)	Biphasic increase ($n = 16$)	Thapsigargin (20 nM)		Oscillations independent of Ca^{2+} ($n = 5$)	Biphasic increase ($n = 8$)
			Before	After		
Average frequency during Ca^{2+}-free period (min^{-1})	0.27 ± 0.02	0.21 ± 0.02	0.22 ± 0.02	0.34 ± 0.03**	1.00 ± 0.14	0.28 ± 0.05
Average spike duration before Ca^{2+} addition (s)	34 ± 4	101 ± 13	50 ± 5	86 ± 13*	35 ± 5	85 ± 11
Loss of amplitude per spike (% of the preceding spike)	6 ± 2	19 ± 3	10 ± 1	36 ± 5**	5 ± 1	23 ± 3
$[Ca^{2+}]_i$ rise upon Ca^{2+} addition (△ ratio)	NA	0.52 ± 0.06	0.23 ± 0.02***		NA[a]	0.61 ± 0.10
Acceleration in spiking frequency (%)	+85	NA	NA		−6	NA

[a] NA, not applicable.

significantly smaller than in cells in which a biphasic $[Ca^{2+}]_i$ rise in response to extracellular Ca^{2+} occurred in the absence of thapsigargin (Table I). Thapsigargin also caused a transient increase of spiking frequency before slowing and eventually terminating the oscillatory Ca^{2+} signal (Fig. 2*C* and Table I).

Involvement of Ca^{2+} Entry in the Generation of $[Ca^{2+}]_i$ Responses to High Concentrations of Ang II—To study the role of extracellular Ca^{2+} during stimulation with high Ang II concentrations, glomerulosa cells were treated with 10 nM Ang II in Ca^{2+}-free medium (no added Ca^{2+}, without EGTA). This hormone concentration usually caused a biphasic $[Ca^{2+}]_i$ increase when added in the presence of Ca^{2+} (Fig. 1*C*). However, under Ca^{2+}-free conditions 10 nM Ang II evoked Ca^{2+} oscillations, although of a different character than those elicited by 100 pM Ang II (Fig. 4 and Table I). Such responses were typically large and rapid Ca^{2+} elevations in which the individual spikes were superimposed and showed a progressive decrease in amplitude. This initial phase was usually followed by base-line oscillations of higher frequency and of variable amplitude and spike duration, depending on the individual cell. The responses of these cells to the addition of Ca^{2+} could be classified as follows. In about 30% of the cells (5 of 15) in which the Ang II-evoked signal stabilized as regular oscillations of high amplitude, narrow spike duration, and high frequency, the response was unaffected by extracellular Ca^{2+} (Fig. 4*A*), being 1.00 ± 0.14 and 0.94 ± 0.14 min^{-1} in the absence and presence of 0.6 mM $[Ca^{2+}]_o$, respectively. In a second group of cells (8 of 15) that showed wide, low frequency spikes of greatly diminished amplitude, the addition of Ca^{2+} caused a biphasic $[Ca^{2+}]_i$ increase (Fig. 4*C*). The remaining two cells showed an "intermediate" response in that the addition of 0.6 mM Ca^{2+} caused a transient run of high frequency oscillations with an elevated interspike $[Ca^{2+}]$-level, followed by a return to high frequency base-line oscillations (Fig. 4*B*).

Effects of Dihydropyridines on Ang II-induced Ca^{2+} Oscillations—The contribution of VSCCs to the regulation of Ca^{2+} oscillations was examined in Ang II-stimulated cells treated with dihydropyridine agonist (BAY K 8644) and antagonist (nifedipine) derivatives. As shown in Fig. 5*A*, BAY K 8644 (10 nM) significantly increased the frequency of Ang II (100 pM)-induced Ca^{2+} oscillations (in 7 of 13 cells) by 86% from 0.43 ± 0.08 to 0.80 ± 0.18 min^{-1} ($p < 0.02$) or changed the oscillatory Ca^{2+} signal to a plateau type response (in 6 of 13 cells, Fig. 5*B*). Conversely, nifedipine (2 μM) significantly decreased the frequency of Ca^{2+} oscillations by 49% from 0.76 ± 0.08 to 0.39 ± 0.05 min^{-1} ($p < 0.02$, $n = 6$ out of 6 cells, Fig. 5*C*). In an additional subset of cells that exhibited Ca^{2+} plateaus even at low Ang II concentrations, these were converted by nifedipine to regular repetitive Ca^{2+} transients ($n = 7$ cells, Fig. 5*D*). In contrast, the plateau components of biphasic Ca^{2+} responses elicited by high Ang II concentrations (10 nM) were not significantly affected by either nifedipine (Fig. 1*C*) or BAY K 8644 (not shown). In the few cells that displayed spontaneous $[Ca^{2+}]_i$ signals in the absence of any stimulus (in the form of elevated, noisy base lines), these were terminated by treatment with nifedipine (Fig. 5*E*), suggesting their dependence on Ca^{2+} entry through VSCCs. The reciprocal effects of BAY K 8644 and nifedipine on $[Ca^{2+}]_i$ signaling following stimulation with a high $[K^+]_o$ (30 mM) to activate VSCCs in glomerulosa cells are shown in Fig. 5*F*.

Contribution of VSCC to Regulation of Ca^{2+} Oscillations by Extracellular Ca^{2+}—To estimate the extent to which Ca^{2+} influx through VSCC contributes to the rise in frequency caused by increases in extracellular Ca^{2+} concentration, nifedipine (2 μM) was added after the restoration of $[Ca^{2+}]_o$ in Ang II (100 pM)-stimulated cells (Fig. 2*A*). Comparison of the oscillatory frequencies observed before and after Ca^{2+} addition and after administration of nifedipine in the presence of external Ca^{2+}, gives an index of the VSCC component of extracellular Ca^{2+}-dependent frequency modulation. Such comparison was achieved by expressing the oscillation frequency as a percentage of that observed in the presence of Ca^{2+} after Ca^{2+} restoration. This analysis showed that while the frequency was decreased by 30% after administration of nifedipine in the Ca^{2+}-restored state it was still significantly higher than that recorded before Ca^{2+} addition (50%, $n = 6$ cells; Fig. 2*A* and Table II). Nifedipine (2 μM) had no effect on the plateau level of $[Ca^{2+}]_i$ when the same experimental protocol was used with a high dose (10 nM) of Ang II (not shown).

Effect of Dihydropyridines on Inositol Phosphate Formation—Since Ca^{2+} influx has been shown to affect $InsP_3$ formation in agonist-stimulated cells (24, 30, 31), we determined whether the effects of dihydropyridines on Ca^{2+} signaling were due to the increased formation of this second messenger. Glomerulosa cells prelabeled with *myo*-[^{3}H]inositol were stimulated with low concentrations (100 or 300 pM) of Ang II in the

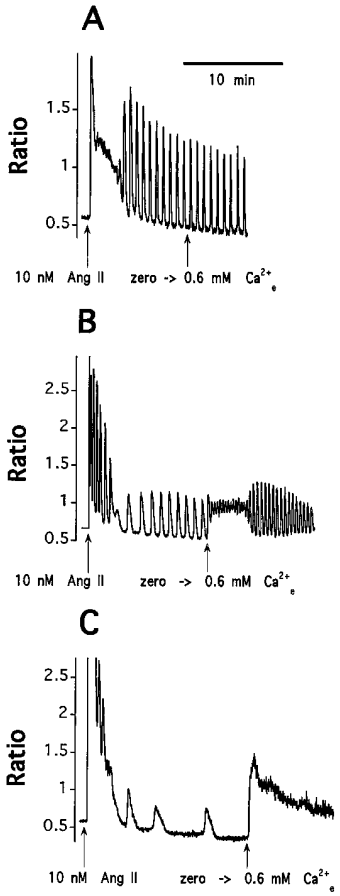

FIG. 4. **Effect of extracellular Ca^{2+} on Ca^{2+} signaling induced by high concentration of Ang II (10 nM).** Glomerulosa cells stimulated with 10 nM Ang II in Ca^{2+}-free medium displayed two typical types of Ca^{2+} oscillations: one with high frequency that was not affected by the addition of extracellular Ca^{2+} (*A*; also compare with Fig. 1, *D* and *E*) and another with a rapid rise in $[Ca^{2+}]_i$ in response to 0.6 mM $[Ca^{2+}]_o$ followed by a nonoscillatory plateau (*C*; see also Fig. 1C). In a few cells, the oscillatory signal was maintained at higher frequency after the admission of extracellular Ca^{2+} (*B*).

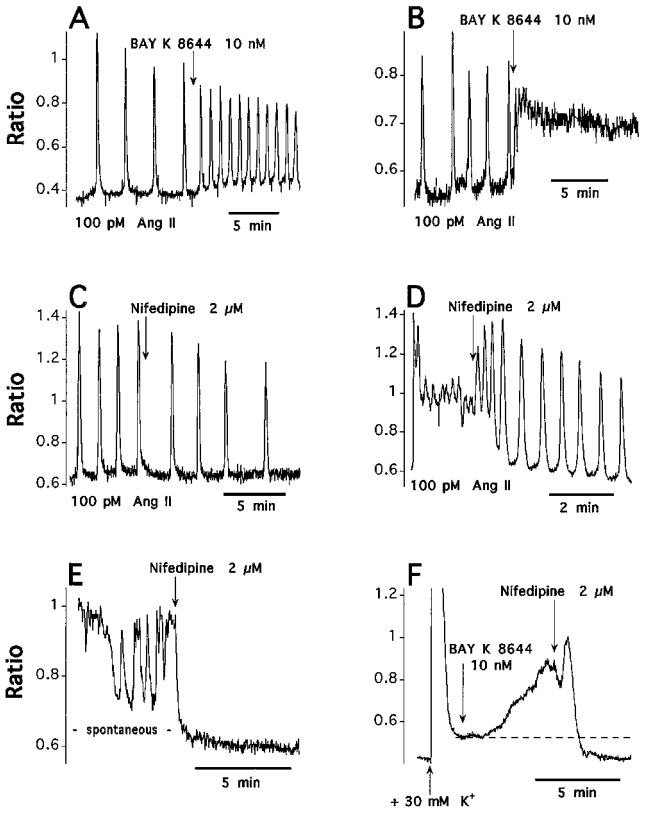

FIG. 5. **Regulation of Ang II-induced Ca^{2+} oscillations by dihydropyridines.** Adrenal glomerulosa cells were stimulated with 100 pM Ang II in normal Ca^{2+} medium to evoke Ca^{2+} oscillations. The addition of BAY K 8644 (10 nM) results in an increase in the frequency of the transients (*A*) or shifts the oscillatory signal to a sustained $[Ca^{2+}]_i$ plateau (*B*). *C*, nifedipine (2 μM) reduces the oscillatory frequency. *D*, highly responsive cells that displayed a plateau-type signal in response to 100 pM Ang II were returned to regular oscillations by nifedipine. *E*, spontaneous irregular elevations in $[Ca^{2+}]_i$ in the absence of agonist occurred in a small subset of glomerulosa cells and were sensitive to nifedipine. *F*, the effects of BAY K 8644 and nifedipine on the extracellular K^+ (30 mM)-induced $[Ca^{2+}]_i$ response in single bovine adrenal ZG cells are shown for comparison with those elicited by Ang II.

presence of 10 mM Li^+ to quantitate the small amount of $InsP_3$ (mostly in the form of its metabolites) that is produced under these conditions. The effects of the dihydropyridines on inositol phosphate responses to 100 pM Ang II were negligible compared with the prominent response elicited by raising the Ang II concentration from 100 pM to 300 pM (Fig. 6). Since the addition of BAY K 8644 and elevation of Ang II concentration from 100 to 300 pM caused a comparable increase in Ca^{2+} oscillatory frequency (86 and 75% increases, respectively), these results indicated that the effects of dihydropyridines on Ca^{2+} oscillations are not attributable to changes in $InsP_3$ production.

DISCUSSION

The characteristics of the oscillatory Ca^{2+} responses of adrenal glomerulosa cells to low doses of Ang II were similar to those of the "base line-spiking" Ca^{2+} oscillations first described in single hepatocytes (32). These oscillations consisted of Ca^{2+} transients separated by long interspike base-line intervals and showed frequency modulation at low agonist concentrations. Higher concentrations of Ang II usually evoked nonoscillatory signals that consisted of a rapid rise to a peak $[Ca^{2+}]_i$ followed by a fall to a lower plateau elevation ("biphasic response"). The rapid initial $[Ca^{2+}]_i$ increase appeared to consist of superimposed Ca^{2+} transients that sometimes could be resolved into partially separated spikes. Individual Ca^{2+} responses showed a degree of heterogeneity, and in many cases low agonist doses initiated regular oscillations that subsequently changed to nonoscillatory plateau increases. Conversely, a small number of cells responded to high dose Ang II stimulation with high frequency oscillations that were maintained for a prolonged period of time.

The basic mechanism of the oscillations observed in cells stimulated by Ca^{2+}-mobilizing hormones is dependent on the $InsP_3$-gated Ca^{2+} release from intracellular stores through the $InsP_3$ receptor-Ca^{2+} channel, and its subsequent reuptake into the same stores. The periodicity of Ca^{2+} release at any given $InsP_3$ concentration is believed to result from the rapid stimulatory and slower inhibitory effects of small and large $[Ca^{2+}]_i$ increases, respectively, on the activity of the $InsP_3$ receptor channel (3, 33). Accordingly, in addition to agonist-induced increases in intracellular $InsP_3$ concentration, changes in $[Ca^{2+}]_i$ have a major impact on the character of Ca^{2+} oscillations. Theoretically, $InsP_3$-induced oscillations can be maintained in a closed system in which there is no net movement of Ca^{2+} through the plasma membrane and only the periodic emptying and refilling of the intracellular Ca^{2+} stores contributes to the cytosolic Ca^{2+} changes (34). However, in real cells the plasma membrane Ca^{2+} pumps and the various Ca^{2+} influx mechanisms make major contributions to the changes in $[Ca^{2+}]_i$. It was previously assumed that the activation of voltage-sensitive Ca^{2+} influx mechanism(s) by periodic depolariza-

TABLE II

DHP-sensitive component of extracellular Ca^{2+}-mediated regulation of Ang II-induced Ca^{2+} oscillations

Comparison of the intertransient intervals (mean ± S.E.) in six individual bovine adrenal glomerulosa cells following stimulation with 100 pM Ang II in Ca^{2+}-free medium, before, and after the addition of 0.6 mM $[Ca^{2+}]_o$ and following the subsequent addition of nifedipine (2 μM). The frequencies are expressed as percentage of that observed in 0.6 mM $[Ca^{2+}]_o$-containing medium (control, 100%). Statistical significance was calculated by analysis of variance with the Duncan test (*, $p < 0.005$ compared with 0.6 mM $[Ca^{2+}]_o$; **, $p < 0.005$ compared with 0.6 mM $[Ca^{2+}]_o$ and $p < 0.05$ compared with Ca^{2+}-free, *F*, nifedipine-sensitive part of the Ca^{2+}-dependent frequency enhancement as percentage of the entire frequency increase; *n*, number of intervals counted in each trace.

Cell	Ca^{2+}-free				0.6 mM $[Ca^{2+}]_o$				Nifedipine (2 μM)				
	n	min^{-1}	%	*s*	*n*	min^{-1}	%	*s*	*n*	min^{-1}	%	*s*	*F*
1	5	0.46	31	131 ± 6	10	1.5	100	40 ± 1	10	0.54	36	112 ± 2	92
2	3	0.18	38	341 ± 5	6	0.48	100	126 ± 4	5	0.41	85	148 ± 11	23
3	5	0.29	67	208 ± 45	6	0.43	100	138 ± 5	7	0.36	84	169 ± 19	50
4	4	0.23	72	256 ± 17	4	0.32	100	188 ± 6	4	0.24	75	250 ± 6	89
5	5	0.28	43	217 ± 11	8	0.65	100	93 ± 6	7	0.48	74	126 ± 6	46
6	4	0.23	45	262 ± 24	7	0.51	100	117 ± 5	6	0.34	67	175 ± 8	61
Mean ± S.E. of six cells		0.28 ± 0.04	49 ± 7	236 ± 28		0.65 ± 0.18	100	117 ± 20		0.40 ± 0.04	70 ± 7	163 ± 20**	60 ± 11

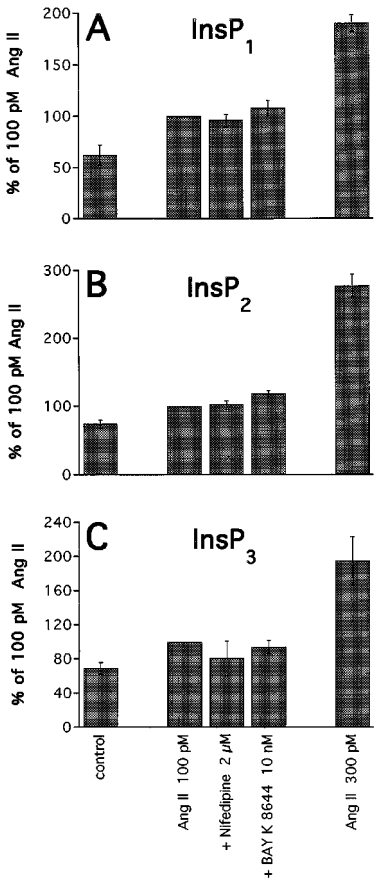

FIG. 6. **Effects of dihydropyridines on the accumulation of inositol phosphates in glomerulosa cells stimulated with 100 pM Ang II.** Bovine adrenal glomerulosa cells prelabeled with [^{3}H]inositol were preincubated with 10 mM Li^+ for 30 min before stimulation with 100 pM or 300 pM Ang II for 30 min. Nifedipine (2 μM), BAY K (10 nM), or medium were added after 8 min of stimulation by 100 pM Ang II. Results are presented as mean ± S.E. of three or four individual experiments, each performed in duplicate. Similar but smaller changes were observed in the absence of Li^+ (data not shown).

tion (action potentials), as well as capacitative Ca^{2+} entry pathways activated by the depletion of intracellular Ca^{2+} stores, cause significant increases in $[Ca^{2+}]_i$. However, recent data suggest that under physiological conditions such Ca^{2+} influx can modulate Ca^{2+} release from internal stores without necessarily changing the average cytosolic Ca^{2+} concentration (11). This concept posits that the limited amount of Ca^{2+} that enters the cell during agonist stimulation does not cause a measurable increase in $[Ca^{2+}]_i$ but rather a local elevation that influences the Ca^{2+} release process by acting specifically at the Ca^{2+}-regulatory site of the $InsP_3$ receptor.

The results of the present study clearly demonstrate that Ca^{2+} influx regulates the frequency of Ca^{2+} oscillations in adrenal glomerulosa cells stimulated by low agonist concentrations. Similar findings have been recently reported in the exocrine avian nasal gland and in HeLa cells (35, 36). However, in glomerulosa cells, nifedipine and BAY K 8644 had a pronounced effect on Ca^{2+} oscillations, suggesting that the extracellular Ca^{2+}-dependent frequency modulation involves dihydropyridine-sensitive VSCCs. Furthermore, the increased oscillatory frequency caused by the addition of Ca^{2+} was not usually accompanied by a significant increase in base-line $[Ca^{2+}]_i$, indicating that the amount of Ca^{2+} entering the cells did not cause an overall increase in the cytosolic Ca^{2+} concentration. Rather, such Ca^{2+} influx changed the sensitivity of the $InsP_3$-dependent triggering process that determines the oscillation frequency. Such a change could result from either an increase in the $InsP_3$ concentration caused by Ca^{2+} influx (24, 31) or local increases of Ca^{2+} at the microdomains adjacent to the Ca^{2+}-regulatory site of the $InsP_3$-receptor/Ca^{2+} channel (3).

Studies in [^{3}H]inositol-labeled glomerulosa cells indicated that the frequency modulation caused by dihydropyridines was not accompanied by corresponding changes in $InsP_3$ production. In cells stimulated with 100 pM Ang II, the addition of BAY K 8644 caused a similar frequency increase as elevation of the Ang II concentration to 300 pM but had no significant effect on inositol phosphate production. These findings suggest that changes in Ca^{2+} concentration due to increased Ca^{2+} influx have an important regulatory function at the level of the $InsP_3$ receptor. In this regard, it is interesting to note that a significant negative correlation exists between the duration of Ca^{2+} spikes and the interspike intervals in cells stimulated with low Ang II concentrations in Ca^{2+}-free medium, so that relatively narrow spikes were accompanied by higher spiking frequency. Since cells that show these narrow spikes do not appear to lose significant amounts of Ca^{2+} during oscillations, this observation indicates that the activity of the SERCA Ca^{2+} pump is an important determinant of the refractory period. This finding is in agreement with recent data obtained in *X. laevis* oocytes, in which overexpression of a SERCA Ca^{2+} pump caused synchronization and increased frequency of oscillations (12).

While regular oscillations were characteristic of most glomerulosa cells stimulated with low agonist concentrations, these usually changed to sustained $[Ca^{2+}]_i$ elevations at higher agonist doses, and in some cases even at low Ang II concentrations. Such nonoscillatory Ca^{2+} responses appeared to be a characteristic of cells in which the intracellular Ca^{2+} pools are

unable to completely refill, with consequently more active capacitative Ca^{2+} influx pathways. This is clearly the case when cells are stimulated with high Ang II concentrations in the presence of Ca^{2+}, where the high levels of $InsP_3$ prevent pool refilling. Cells exposed to high concentrations of Ang II are still able to oscillate in Ca^{2+}-deficient medium, partly due to a smaller $InsP_3$ increase (24) and a lesser Ca^{2+} load in the cytosol due to the lack of Ca^{2+} influx. A similar extracellular Ca^{2+}-dependent switch from oscillatory to nonoscillatory response was seen in some cells stimulated at low Ang II concentration after the addition of dihydropyridines. Thus, the addition of BAY K 8644 sometimes converted the oscillatory response to a sustained plateau, while nifedipine reestablished regular oscillations in cells that showed sustained $[Ca^{2+}]_i$ increases.

The heterogeneity of the $[Ca^{2+}]_i$ responses to the addition of external Ca^{2+} in Ang II-stimulated cells also shed some light on the conditions under which Ca^{2+} oscillations are not maintained in the presence of Ca^{2+}. Thus, the biphasic Ca^{2+} increase with no oscillations was a characteristic response to Ca^{2+} addition of cells that showed broad spikes and/or rapidly decreasing spike amplitudes when stimulated with Ang II in Ca^{2+}-free medium. On the other hand, cells with narrow and regular spikes responded to the addition of Ca^{2+} with an increase in oscillatory frequency but could behave like the former cells after minor inhibition of their SERCA Ca^{2+} pump by thapsigargin. These observations also implicate the Ca^{2+} reuptake mechanism(s), and its ability to restore basal Ca^{2+} levels and refill the Ca^{2+} pools, as a major prerequisite for the maintenance of regular oscillations in the presence of Ca^{2+}. Under conditions of pool depletion, the major form of Ca^{2+} entry is the capacitative Ca^{2+} influx pathway, as evidenced by the large capacitative refilling event observed in such cells after the readdition of Ca^{2+}. The minor effects of dihydropyridines on the sustained $[Ca^{2+}]_i$ elevations in these cells are consistent with the notion that the amount of Ca^{2+} entering through VSCCs during Ang II stimulation is not sufficient to produce a major increase of the average cytosolic Ca^{2+} level.

In summary, the present study demonstrates that in adrenal glomerulosa cells stimulated by low Ang II concentrations, Ca^{2+} entry through VSCCs acts as a major regulator of the cytosolic Ca^{2+} response, primarily by modifying the characteristics of the Ca^{2+} oscillations. At higher agonist concentrations, or when intracellular Ca^{2+} pools are subject to depletion, the capacitative entry pathway, which is a major contributor to the plateau increase of $[Ca^{2+}]_i$, is activated with no further oscillations. Local changes in the Ca^{2+} concentration at the Ca^{2+}-regulatory site of the $InsP_3$ receptor Ca^{2+} channel probably represent the mechanism through which extracellular Ca^{2+} affects Ca^{2+} oscillations.

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